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Selection and Phenotypic Characterization of Nonhemagglutinating Mutants of *Porphyromonas gingivalis*

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To further investigate the relationship between fimbriae and the hemagglutinating adhesin HA-Ag2 of *Porphyromonas gingivalis*, three spontaneous mutants of the type strain ATCC 33277 were selected by a hemadsorption procedure. They were characterized for hemagglutination, trypsin-like and lectin-binding activities, and hydrophobicity and for the presence of fimbriae. The presence of the 42-kDa (the fimbrilin subunit) and the 43- and 49-kDa (the HA-Ag2 components) polypeptides was investigated by immunoblotting using polyclonal and monoclonal antibodies directed to fimbriae and to the hemagglutinating adhesin HA-Ag2. Cells from two of the three mutants (M1 and M2) exhibited no or little hemagglutination activity and very low trypsin-like activity and did not show the 43- and 49-kDa polypeptides. Abnormal fimbriation in M1 was deduced from the following observations of cells grown for 18 h: absence of the 42-kDa polypeptide and of a 14-kDa polypeptide and no fimbriae visible on electron micrographs. While the cells of mutant M2, irrespective of the age of the culture, were found to lack the 43- and 49-kDa polypeptides and hemagglutination activity, the supernatants of cultures grown for 72 h had high hemagglutination and trypsin-like activities and revealed the presence of the 42-, 43-, and 49-kDa polypeptides. This suggests that M2 may be missing some molecules which anchor the components to the cell surface. Mutant M3 showed levels of activities similar to those of the parental strain but lacked the 43-kDa polypeptide. Other pleiotropic effects observed for the mutants included loss of dark pigmentation and lower hydrophobicity. The data from this study fuel an emerging consensus whereby fimbriation, hemagglutination, and proteolytic activities, as well as other functions in *P. gingivalis*, are intricate.

Porphyromonas gingivalis, a gram-negative anaerobic bacterium, has been associated with different forms of periodontal diseases. Several properties of this microorganism, in particular hemagglutination and trypsin-like activities, are thought to contribute to its pathogenicity. *P. gingivalis* possesses various cell surface components that mediate binding to epithelial cells (30, 41), erythrocytes (13, 30, 31), and other bacterial species (8, 20). Fimbriae in particular have been reported to play an important role in adherence to various surfaces, facilitating the initial interaction between the bacteria and the host (14). Early claims that the fimbriae were responsible for hemagglutination activity (HA) (30) have been contested (for a review see reference 24). More recently, Ogawa and Hamada (29) attributed an HA to fimbriae and to synthetic peptide segments of the fimbrial protein from *P. gingivalis* 381. These observations indicate that the reports regarding hemagglutinins of *P. gingivalis* and the role of fimbriae in the hemagglutination process are still controversial. In addition, several studies (9, 11, 18, 20, 27, 28) have presented evidence that adherence and protease activities involved the same molecule.

Recent work in our laboratory has focused on an antigenic, structural, and functional relationship between the fimbriae and the hemagglutinating adhesin HA-Ag2 of *P. gingivalis* ATCC 33277 (4). By using monoclonal antibodies (MAbs) produced against these two cell surface components, an intimate association was demonstrated, and a hypothesis for the structure of the *P. gingivalis* fimbriae and their association with the hemagglutinating adhesin HA-Ag2 was proposed (5). To test our hypothesis, the present study was designed to obtain mutants of *P. gingivalis* devoid of HA, using a hemadsorption procedure. Characterization of these mutants for their immunoreactivity with anti-fimbria and anti-HA-Ag2 antibodies, by immunoblotting and immunoelectron microscopy, as well as for their hydrophobicity and trypsin-like and lectin-binding activities contributed to the elucidation of the functional relationship between the fimbriae and the hemagglutinating adhesin HA-Ag2 and in addition revealed a strong relationship between the HA and the trypsin-like activities in *P. gingivalis.*

MATERIALS AND METHODS

Abbreviations. Abbreviations used are as follows: HA, hemagglutination activity; MAbs, monoclonal antibodies; VCS, vesicle-containing supernatant; VFS, vesicle-free supernatant.

Bacterial strain and growth conditions. The type strain *P. gingivalis* ATCC 33277 was grown in Todd-Hewitt broth enriched with hemin (10 μg/ml) and vitamin K_1 (1 μg/ml), in an anaerobic atmosphere (80% N_2, 10% H_2, 10% CO_2) at 37°C. Cultures were maintained on the same medium containing 1.5% agar and 2% laked human blood. Cells were either grown for 18 to 20 h (young culture) or 72 h (old culture). Unless otherwise indicated, cells were harvested by centrifugation (8,000 × *g* for 20 min) and washed twice in phosphate-buffered saline (PBS, 10 mM, pH 7.2). The vesicle-containing supernatant (VCS) was obtained by centrifugation (10,000 × *g* for 30 min) of the *P. gingivalis* culture. The vesicle-free supernatant (VFS) resulted from the ultracentrifugation (100,000 × *g* for 60 min) of the supernatant obtained above. Both VCS and VFS were concentrated nine times by ultrafiltration through a membrane with a molecular weight cutoff of 10,000.

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Isolation of mutants by hemadsorption. The procedure of Ørskov et al. (32) was used to isolate mutants of *P. gingivalis* deficient in HA. A suspension of *P. gingivalis* in sterile PBS containing about 2×10^9 cells per ml was mixed with 10 ml of a 1% sheep erythrocyte suspension. The mixture was incubated for 1 h at 37°C with gentle agitation before centrifugation at low speed ($200 \times g$ for 1 min). The supernatant was removed and subjected two additional times to the hemadsorption procedure. After the last centrifugation, 50-μl samples of the supernatant were spread onto agar plates which were incubated anaerobically for 7 days. The bacterial colonies were screened for their ability to bind erythrocytes, and those that were found to be negative were physiologically characterized and compared with the parent strain by using a commercial identification system (ID 32 A; BioMerieux).

Screening for hemadsorption-negative mutants. Bacterial colonies selected by the above hemadsorption procedure were screened for their ability to bind sheep erythrocytes. Nitrocellulose discs (pore size, 0.45 μm; Schleider & Schuell, Inc.) were wetted in PBS and placed on the surface of the agar plate. To ensure complete contact between the disc and the plate, the disc was lightly pressed against the plate with a sterile bent glass rod, and the plate was incubated at 37°C for 30 min. The discs were then carefully removed from the agar plates, and 10 ml of 1% gelatin in PBS was added to block unoccupied sites on the nitrocellulose membranes, which were gently agitated at room temperature for 1 h. The gelatin was discarded, and the discs were incubated with 10 ml of a 5% sheep erythrocyte suspension for 1 h at room temperature. After two rinses in PBS–0.05% Tween 20, the discs were incubated in the presence of a mouse antibody directed against sheep erythrocytes (1/250 dilution) in PBS containing 1% gelatin. Unbound antibodies were removed by washing for 30 min in PBS–0.05% Tween 20. Bound antibodies were detected by goat anti-mouse immunoglobulin G (IgG) and IgM (1/3,000 dilution) conjugated to alkaline phosphatase (Jackson ImmunoResearch Laboratories). Hemadsorption-negative colonies were picked from the original petri plates by reference to the nitrocellulose prints, and subcultured on fresh THB agar plates.

Production of polyclonal antibodies and MAbs. A monospecific antiserum, WL305, was produced against the hemagglutinating adhesin HA-Ag2 of *P. gingivalis* by immunization of rabbits with the corresponding immunoprecipitate obtained by crossed immunoelectrophoresis (CIE) as previously described (3). The immunoprecipitate was dissolved in 2 ml of PBS, mixed with 2 ml of Freund's complete adjuvant, and injected intramuscularly into each hind limb and at eight sites on the back. A second injection with the HA-Ag2 precipitate was given after 2 weeks intradermally at several sites on the back. Two similar booster injections were given intramuscularly at weekly intervals. The animals were bled the following week, and the antisera were tested for antibody titers by immunoblotting against bacterial extracts.

A fimbria-specific antiserum, XL894, was produced by immunizing rabbits with the fimbria immunoprecipitate, obtained by CIE, following the protocol described above for antiserum WL 305.

MAbs against HA-Ag2 were produced by immunizing mice with immunoprecipitates excised from replicate CIE gels. Characterization of these MAbs and epitope mapping of HA-Ag2 were previously described (6, 7). MAbs against native fimbriae were produced by immunizing mice with purified fimbriae. Characterization of these MAbs and epitope mapping of fimbriae have been described elsewhere (4).

Determination of HA. Hemagglutination was measured in round-bottom microtiter plates. Briefly, 75 μl of PBS was added to each well, and then 75 μl of the bacterial fractions (bacterial cells, VCS or VFS) was added to the first well and diluted serially. Finally, 75 μl of 1% washed sheep erythrocytes was added to each well and the plates were incubated for 2 to 4 h at 4°C. Hemagglutination was assessed visually, and the reciprocal of the highest dilution displaying a positive agglutination of erythrocytes was recorded.

Determination of trypsin-like activity. Trypsin-like activity was determined by using *N*-α-benzoyl-DL-arginine *p*-nitroanilide (BA*p*NA) as the substrate (Sigma Chemical Co., St. Louis, Mo.). Briefly, bacterial fractions (100 μl; cells, VCS or VFS) were mixed with 100 mM Tris—2.5 mM dithiothreitol buffer (600 μl) and 10 mM of the synthetic substrate (50 μl). The incubation period for the assay was 60 min at 37°C. The reaction was stopped by adding 4 M acetic acid, and the A_{405} was measured. Uninoculated medium and buffer were used as controls.

Determination of lectin-binding activity. Lectin-binding activity of whole cells was determined by a dot blotting procedure. Six horseradish-peroxidase-coupled lectins were used: LOTUS A (*Tetragonolobus purpureas*; specific for fucose residues), concanavalin A (*Canavalia ensiformis*; for specific mannose residues), RCA-1 (*Ricinus communis*; specific for galactose residues), PNA (*Arachis hypogaea*; specific for galactose residues), SBA (*Glycine max*; specific for *N*-acetyl-D-galactosamine and galactose residues), and WGA (*Triticum vulgaris*; specific for *N*-acetyl-D-glucosamine residues). Except for two minor modifications (cell suspension at $A_{660} = 2.0$ and horseradish peroxidase-conjugated lectin concentration at 5 μg/ml), the assay was identical to one already described (10). A positive reaction was indicated by a strong purple spot and a weak reaction was indicated by a light purple spot, whereas a negative reaction gave no coloration.

Determination of cell hydrophobicity. The method for measuring bacterial adherence to hydrocarbon was as previously described (38). Briefly, the bacteria were grown at 37°C for 24 h in enriched Todd-Hewitt broth, washed three times, and resuspended in buffer to an optical density (400 nm) of 1.0. A sample (1.2 ml) of bacterial suspensions was placed in a tube, and hexadecane (200 μl) was added. The tubes were mixed for 2 min at room temperature using a vortex mixer. The tubes were left to stand for 15 min, and the A_{400} of the aqueous phase was measured. The percent hydrophobicity was calculated as follows: $[(A_{400}$ before mixing $- A_{400}$ after mixing$/A_{400}$ before mixing$)] \times 100$. Each isolate was assayed twice, and the values obtained were averaged.

SDS-PAGE and immunoblotting analysis. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out using the buffer system of Laemmli (17) at a constant voltage (200 V). Cells were pretreated with 10% trichloroacetic acid at 4°C overnight. The samples were then solubilized in dissociating buffer containing 2% SDS and 5% β-mercaptoethanol and heated at 100°C for 5 min. One gel was fixed and silver stained (1), whereas polypeptides present in a second gel were transferred onto a polyvinylidene difluoride (PVDF) membrane by electrophoresis (43). Immunological detection on PVDF membranes was carried out at room temperature. Unoccupied sites on membranes were blocked by incubating the membranes for 1 h in Tris-buffered saline (TBS; 20 mM Tris HCl [pH 7.5], 500 mM NaCl) containing 1% gelatin. The membranes were then incubated for 2 h with the primary antibody (rabbit antiserum or MAbs) diluted (1:1,000) in TBS–1% gelatin. Unbound antibodies were removed by washing the blots for 30 min in TBS containing 0.05% Tween 20. Bound antibodies were detected by either goat anti-rabbit or goat anti-mouse IgG and IgM conjugated with biotin (1:5,000) and revealed with alkaline phosphatase-conjugated streptavidin (1:3,000) (Jackson ImmunoResearch Laboratories). The enzyme reaction product was developed with NBT/BCIP (nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate; Sigma) as the substrate. The reaction was stopped by washing the membranes in distilled water.

Immunoelectron microscopy. Ten microliters of a bacterial suspension was deposited on Formvar-coated nickel grids at room temperature for 30 min. To block unoccupied sites, grids were incubated in PBS containing 1% bovine serum albumin (BSA) for 20 min. After rinses in PBS and distilled water, the grids were incubated with MAbs diluted (1:200) with PBS containing 0.5% BSA for 30 min at room temperature. After several rinses in PBS and distilled water, the grids were incubated with goat anti-mouse IgG conjugated to 10-nm-diameter colloidal gold particles (Sigma) diluted (1:20) in PBS containing 1% gelatin and 1% BSA for 30 min at room temperature and rinsed again. Finally, samples were negatively stained with 1% phosphotungstic acid for 10 s. Specimens were examined with a JEOL 2000 transmission electron microscope operating at 80 kV.

Protein determination. Protein determination was performed by the method of Lowry et al. (21) using BSA (Sigma) as the standard.

RESULTS

Isolation of mutants and hemagglutination. The hemadsorption procedure, which is based on sedimentation of erythrocyte-bacterium aggregates, was used to enrich a culture for bacteria which did not adhere to sheep erythrocytes. Spontaneous mutants of *P. gingivalis* ATCC 33277 were isolated by this method. Seventeen hemadhesion-negative mutants were detected on blood agar plates, and three of them, designated M1, M2, and M3, were chosen for further studies. Two mutants, M1 and M2, appeared as light beige colonies, whereas M3 formed beige to brown colonies and recovered a dark brown pigmentation when incubated more than 8 days. This first observation suggests that at least a difference in hemin acquisition and storage exist between these mutants. Except for the trypsin-like activity, which was significantly lower in M1 and M2, all three mutants were physiologically identical to the parent strain when identified by ID 32 A, a commercial identification system. When whole cells, as well as VCS and VFS from young (18 h) and old (72 h) cultures, were tested for hemagglutination, several differences could be seen (Table 1). On the whole, fractions from old cultures possessed stronger activity than those from young cultures. In young cultures, no HA was obtained with any preparation of M1 and M2; only 33277 and M3 cells (the latter to a much smaller extent) did hemagglutinate. With older cultures, cells of mutant M1 showed a very weak hemagglutination whereas cultures of M2 did not hemagglutinate at all but exhibited strong HA both in VCS and VFS (Table 1). The VCS of young cultures of M3 exhibited a stronger HA than the same preparation of the parent strain. VFS from young cultures did not show activity for any of the tested isolates, including the parent strain.

SDS-PAGE analysis and immunoreactivity. Comparison of SDS-PAGE protein profiles of whole-cell lysates of the mu-

TABLE 1. Hemagglutination and trypsin-like activities

Fraction and strain	Hemagglutination[a]		Trypsin-like activity[b]	
	Young culture (18 h)	Old culture (72 h)	Young culture (18 h)	Old culture (72 h)
Cells				
ATCC 33277	32	32	0.526	1.02
M1	0	2	0.019	0.086
M2	0	0	0.003	0.035
M3	8	16	0.582	0.765
VCS				
ATCC 33277	4	64	0.403	0.640
M1	0	0	0.025	0.214
M2	0	64	0.187	1.322
M3	32	128	0.802	0.902
VFS				
ATCC 33277	0	2	0.351	0.198
M1	0	0	0.026	0.098
M2	0	128	0.192	1.054
M3	0	2	0.767	0.621

[a] Reciprocal of the highest dilution displaying a positive agglutination of erythrocytes.
[b] Optical density at 405 nm.

tants with that of the wild-type parent revealed no major differences between 33277 and M3 (Fig. 1, lanes 1 and 4). Mutants M1 and M2 (lanes 2 and 3) were both similar in that several of their bands were less intense when compared with those for 33277 and M3, in particular those at 68 and 50 kDa. The silver-stained pattern of M1 (Fig. 1, lane 2) also revealed the absence of the 42-kDa polypeptide as well as many bands below 28 kDa.

An immunoblot assay with cells from young cultures of the three mutants using antiserum XL894 (anti-fimbriae) revealed the absence of the 42-kDa band, the fimbrial subunit, in M1 (Fig. 2, lane 3), which was present in the cell lysates of the other two mutants as well as in 33277. However, older cells of M1 exhibited the 42-kDa band (Table 2). Additional bands with a molecular mass higher than 60 kDa were observed in M1 but not in M2, M3, and the parent strain.

Reactivity of antiserum WL305 (anti-HA-Ag2, the hemagglutinating adhesin) against cells from young cultures of the three mutants revealed the absence of both the 43- and 49-kDa polypeptides (the two components of HA-Ag2) in the cell lysates of M1 and M2 (Fig. 3, lanes 2 and 3) and the presence of the 49-kDa polypeptide in M3 (Fig. 3, lane 4). The parent strain showed the presence of the 43- and 49-kDa (the two components of HA-Ag2) and the 42-kDa (the fimbrial subunit) bands. Polyclonal antibodies and MAbs were also used to detect the 42-, 43-, and 49-kDa bands in VCS and VFS from 33277 and from the three mutants. The two components of HA-Ag2 were found to be absent in VCS of M1 (as previously reported for young cells) but in contrast were both present in M2 (Table 2). Similar results were observed for VFS. No differences were found between young and old cultures.

Trypsin-like activity. When cells from young and old cultures were assayed for trypsin-like activity the most striking result was the absence of any activity in mutants M1 and M2 (Table 1). The activity of M3 was roughly similar to that of the wild-type strain. However, VCS as well as VFS from old cultures of mutant M2 showed very high activity against the substrate. Mutant M1 had no activity in any of the fractions tested.

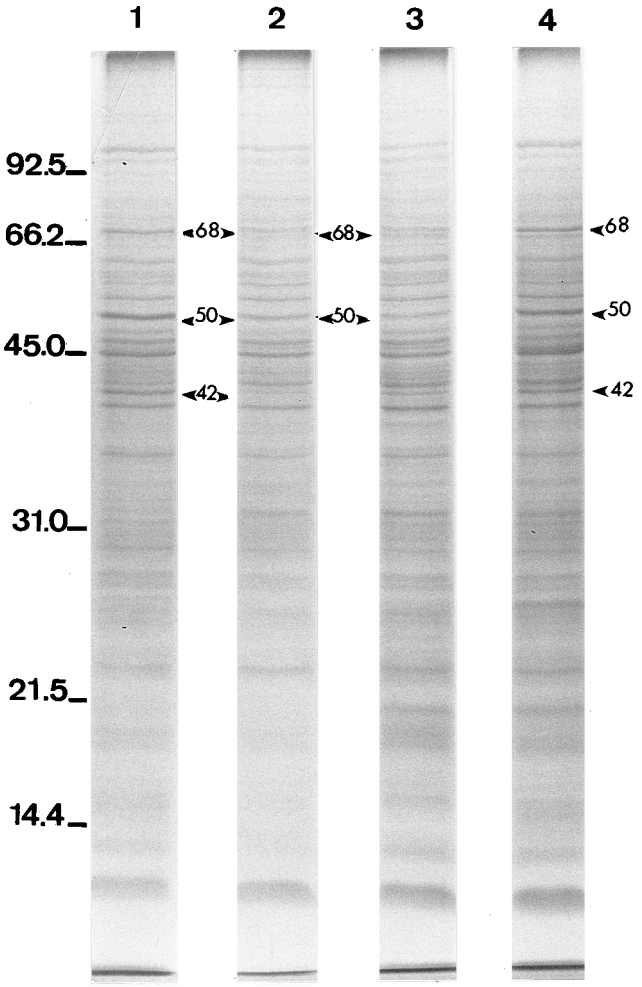

FIG. 1. Silver stained SDS-polyacrylamide gel of cell lysates of the parent strain *P. gingivalis* ATCC 33277 (lane 1) and the three mutants M1 (lane 2), M2 (lane 3), and M3 (lane 4). Low-molecular-weight markers are indicated on the left.

Mutant M3 appeared to have more activity in VCS and VFS as compared with the parent strain.

Lectin-binding activity and cell hydrophobicity. While the parent strain, M1, and M3 had a rather weak ability to bind SBA, mutant M2 possessed a strong binding activity for this lectin (Table 3). The other five lectins reacted in a similar fashion with the parent strain and the three mutants. The hydrophobicity of mutants M1 and M2 was about seven-fold less than that of the wild-type, while that of mutant M3 decreased slightly (Table 3).

Immunoelectron microscopy. Immunolabelling of cells of 33277 and M3 with anti-fimbria and anti-HA-Ag2 MAbs revealed the same appendages previously observed (4, 7) approximately 50 to 100 nm wide and extending up to 3 μm long and attributed to the fimbria-HA-Ag2 complex. An example of such results is presented in Fig. 4A and B. Cells of M2 were lightly labelled with the MAbs (data not shown); however, a dense immunolabelling was observed on free appendages detached from M2 cells (Fig. 4C). No labelling was observed on M1 cells with either the anti-fimbria or anti-HA-Ag2 MAb (Fig. 4D).

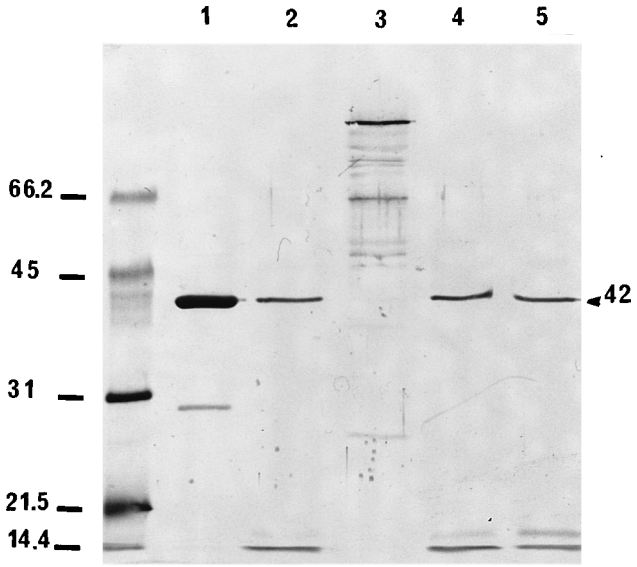

FIG. 2. Immunoblot reactivity of the fimbria-specific antiserum XL 894 with cell lysates of *P. gingivalis* ATCC 33277 and its three mutants, M1, M2, and M3. Lane 1, native fimbriae of *P. gingivalis* ATCC 33277 as a positive control; lane 2, *P. gingivalis* ATCC 33277; lane 3, mutant M1; lane 4, mutant M2; lane 5, mutant M3. Low-molecular-weight markers are indicated on the left.

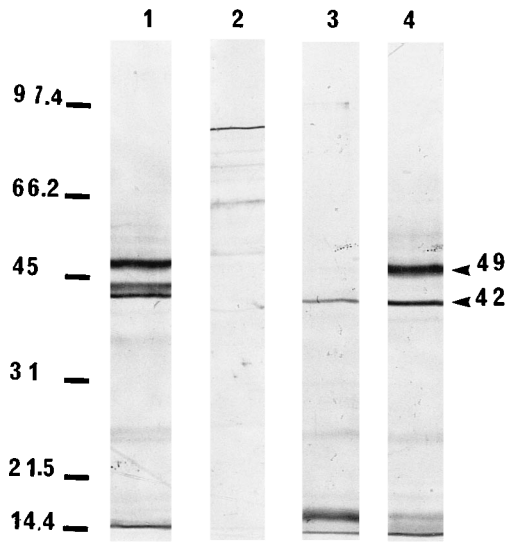

FIG. 3. Immunoblot reactivity of the HA-Ag2-specific antiserum WL 303 with cell lysates of *P. gingivalis* ATCC 33277 and its three mutants, M1, M2, and M3. Lane 1, *P. gingivalis* ATCC 33277; lane 2, M1; lane 3, M2; lane 4, M3. Low-molecular-weight markers are indicated on the left.

DISCUSSION

Hemagglutination is a distinctive characteristic of *P. gingivalis* (19, 23, 24), but several characteristics of this activity are still a matter of debate. For instance, it is not known whether *P. gingivalis* possesses one or more than one hemagglutinin since multiple, and occasionally contradictory, characteristics of hemagglutinins have been reported (2, 13, 23, 31, 37). Also, the exact role of fimbriae in hemagglutination remains controversial (24). Extensive immunochemical characterization of the fimbriae and the hemagglutinating adhesin HA-Ag2 in our laboratory have suggested strong antigenic, structural, and functional relationships between these two components of the *P. gingivalis* cell surface (4). Our results (5) indicated that (i) the smallest structural subunit of fimbriae is a 14-kDa polypeptide, the polymerization of which strongly favors the formation of a 42-kDa trimer, the more stable building block of the fimbrial rod; and (ii) the polymeric structural unit of fimbriae must be complexed with the adhesin to confer HA on the bacterial cell. To further characterize the relationship between the fimbriae and the hemagglutinating adhesin HA-Ag2, we selected spontaneous mutants of the type strain ATCC 33277 by a hemadsorption procedure. Two nonhemagglutinating mutants, M1 and M2, were obtained. No or little HA was found to be associated with any of the tested fractions of M1. Cells from 18-h cultures of this mutant were missing the 42-, 43-, and 49-kDa polypeptides as well as a 14-kDa polypeptide. Since the 42-kDa fimbrilin is actually a trimer of a 14-kDa polypeptide (5), the absence of the 14-kDa polypeptide in M1 correlates well with the absence of the 42-kDa polypeptide and suggests that, at this stage, the fimbriation of M1 is abnormal. Indeed, the electron microscopic observation (Fig. 4) revealed an absence of fimbriae. The loss of HA, which correlates well with the absence of both fimbriae and the hemagglutinating adhesin HA-Ag2 in M1, is a confirmation that an association between fimbriae and HA-Ag2 is necessary to impart HA to the cells. However, our finding in M1 cells (young cultures) of high-molecular-weight proteins antigenically related to fimbriae (Fig. 3) and the detection of the 42-kDa polypeptide in cells and supernatants from old cultures (Table 2) are difficult to reconcile with our postulate. Indeed, the high-molecular-weight proteins may be similar to those reported by other

TABLE 2. Presence of immunoreactive bands upon reaction with antifimbria and anti-hemagglutinating adhesin MAbs

Fraction and strain	Young culture (18 h)			Old culture (72 h)		
	42 kDa	43 kDa	49 kDa	42 kDa	43 kDa	49 kDa
Cells						
ATCC 33277	+	+	+	+	+	+
M1	−	−	−	+	−	−
M2	+	−	−	+	−	−
M3	+	−	+	+	−	+
VCS						
ATCC 33277	+	+	+	+	+	+
M1	+	−	−	+	−	−
M2	+	−	+	+	+	+
M3	+	−	+	+	−	+
VFS						
ATCC 33277	+	+	+	+	+	+
M1	+	−	−	+	−	−
M2	+	+	+	+	+	+
M3	+	−	+	+	−	+

TABLE 3. Lectin-binding activity and hydrophobicity

Strain	Lectin[a]						Hydrophobicity[b] (%)
	Lotus A	ConA	RCA	PNA	SBA	WGA	
ATCC 33277	−	−	−	+	+/−	+	51
M1	−	−	−	+	+/−	+	6
M2	−	−	−	+	+	+	9
M3	−	−	−	+	+/−	+	40

[a] −, no reaction; +/−, weak reaction; +, strong reaction.
[b] Mean of two assays.

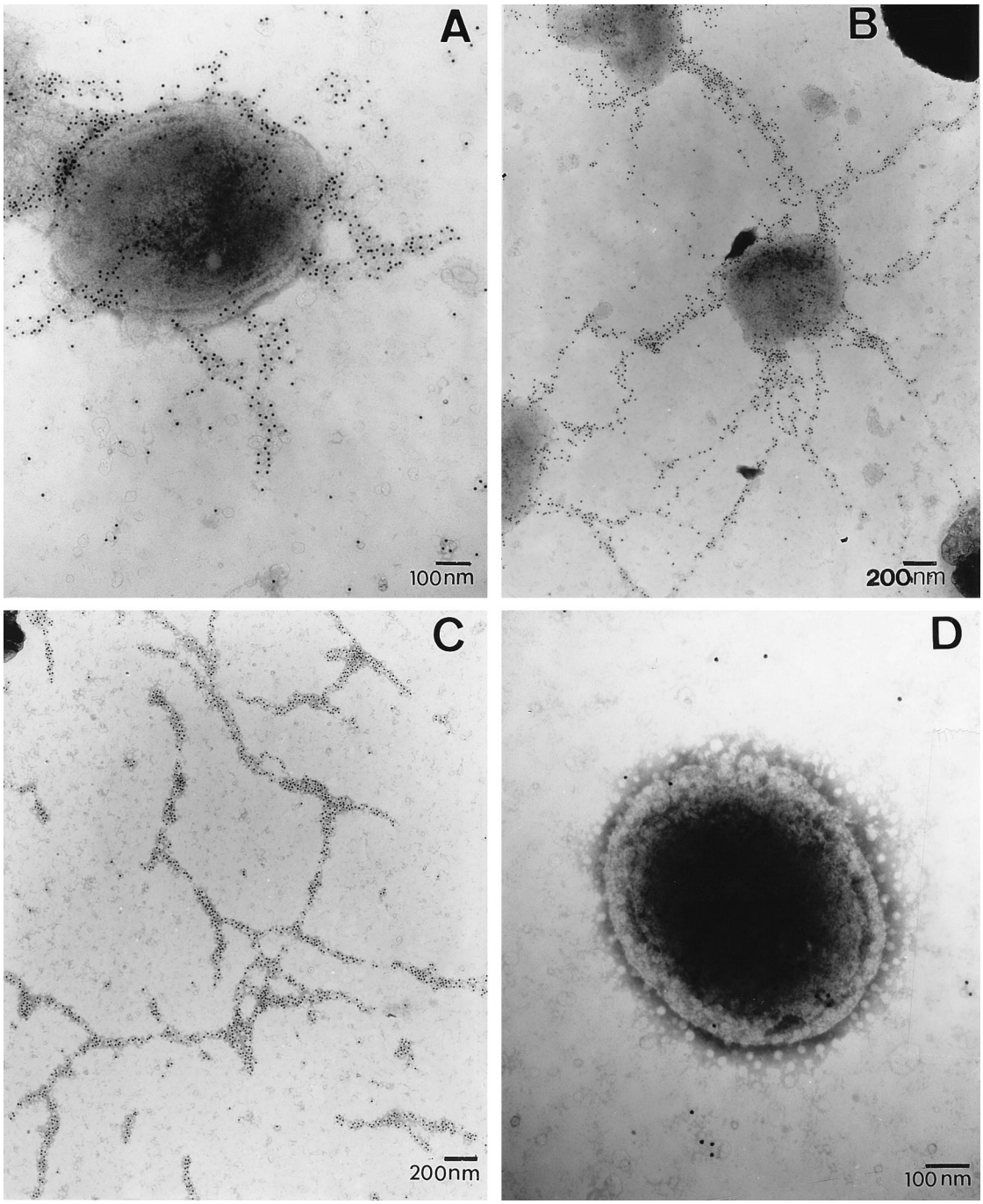

FIG. 4. Immunolocalization of fimbriae on the cell surface of mutant M3 (A and B), M2 (C), and M1 (D) by electron microscopy. Bacterial cells were successively incubated with a fimbria-specific MAb and colloidal gold-conjugated goat anti-mouse immunoglobulin and negatively stained with 1% phosphotungstic acid.

investigators (42, 44) who associated them with polymeric forms of the fimbrial subunit. Alternatively, these immunoreactive bands could be attributed to the loss of a processing enzyme playing a role in the posttranscriptional maturation of fimbrilin. The proteinaceous material of fimbriae, as detected by its antigenicity, would be synthesized by the mutant but its maturation, transmembrane transport, or assembly would be perturbed.

Cells of mutant M2 were found to be devoid of the 43- and 49-kDa polypeptides and had no HA. However, supernatants from 72-h cultures of M2 showed high hemagglutination titers and revealed the presence of the 42-, 43-, and 49-kDa polypeptides. Results with mutant M2 still reinforce the hypothesis of the association of HA with the simultaneous presence of both fimbriae and HA-Ag2. In addition, the strong HA observed in VCS and VFS, which contain all constituents spontaneously released from the cells, suggests that M2 may be missing some molecule which anchors the components to the cell surface. This mutant also lacked hemagglutination and trypsin-like activities in VFS from young cultures, and cells from older cul-

tures expressed strong activities. We propose that the molecules responsible for these activities would remain primarily intracytoplasmic or in the periplasmic space in intact cells of young cultures but would be released after lysis of the cells in old cultures.

Mutant M3 showed activities similar to those of the parental strain and revealed on immunoblots the presence of the 42-kDa (fimbriae) and the 49-kDa (HA-Ag2) polypeptides only. In this regard, mutant M3 should be considered a 43-kDa-band mutant only. The HA observed in M3 may be explained in two ways. First, hemagglutination could be attributed to another hemagglutinin since the possibility that more than one *P. gingivalis* component may function as a hemagglutinin must be considered (19, 24). Second, the 43-kDa polypeptide defined immunochemically together with the 49-kDa polypeptide (23) as a component of the hemagglutinating adhesin HA-Ag2 could not be essential in the HA of *P. gingivalis.*

To summarize, most characteristics of the three mutants reported here corroborate our hypothesis (5) of the structural and functional relationship between fimbriae and the hemagglutinating adhesin in the HA of *P. gingivalis*. Support for our hypothesis can be found in the following clues: (i) absence of fimbriae and HA-Ag2 (the case of M1 cells from young cultures) correlates with the absence of HA; (ii) the presence of the fimbriae only is not sufficient to induce hemagglutination (the case for cells in young cultures of M2), but the presence of both fimbriae and HA-Ag2 induces this activity (the case of VCS and VFS from old cultures of M2); and (iii) HA occurs when fimbriae and the 49-kDa component of HA-Ag2 are present (the case of young and old cultures of M3). Clearly, obtaining an additional mutant that possesses the 49-kDa polypeptide and lacks the fimbriae, as verified with whole cells and culture supernatants, would be of great help in resolving the structural and functional relationship between fimbriae and HA-Ag2.

P. gingivalis also possesses several proteolytic activities, and their association with other activities remains equivocal (35). Nishikata et al. (28) were the first to propose a bifunctional cell surface component responsible for the hemagglutination and trypsin-like activities of *P. gingivalis*, whereas Shah et al. (39) reported independent molecular identity of the hemagglutinin and the cysteine protease of *P. gingivalis*. Our results demonstrated that mutants deficient in HA also showed decreased trypsin-like activity and that bacterial fractions containing high HA exhibited concomitant strong trypsin-like activity. Several studies have reported a close association between hemagglutination and proteolytic activities (11, 15, 27, 33, 34, 36). Hoover et al. (11) reported that trypsin-like protease-deficient mutants obtained by nitrosoguanidine mutagenesis exhibit a concomitant reduction in HA. The authors extended the results of Nishikata et al. (28) by demonstrating that inhibitors and enhancers of the trypsin-like activity had similar effects on the HA. In another study, trypsin-like protease-deficient mutants were found to produce light-pigmented colonies and showed a decreased adhesion to and coaggregation with *Actinomyces viscosus* (20). Grenier (9) showed that strains of *P. gingivalis* with high trypsin-like activity attach in higher numbers to erythrocytes and epithelial cells than bacteria with low levels of activity.

Our mutants also revealed simultaneous loss of pigmentation and lectin-binding activity as well as decreased hydrophobicity. These pleiotropic mutants were isolated using a selective procedure based on the capacity of *P. gingivalis* to adhere to erythrocytes, a physiological activity natural to bacterial life, as indicated by the fact that this event has been correlated with the acquisition of iron (22, 39, 40). Surprisingly, other mutants selected by mutations affecting known protease genes (12, 16, 26) or obtained by random mutagenesis (11) have revealed the same pleiotropic effects. Nakayama et al. (26) described an arginine-specific cysteine proteinase (RGP)-defective mutant with few fimbriae and low amounts of 43- and 45-kDa polypeptides and concluded that the RGP proteinase contributes to fimbriation, degradation of host proteins, and hemagglutination. In another study (25), the same authors reported that hemagglutination and RGP activities were encoded by the same genes. Kuramitsu et al. (16) have reported a cysteine protease mutant defective in the *rgp-1* gene, with reduced capacity to interact with gram-positive bacteria and epithelial cells, altered fimbriation, and reduced 43-kDa fimbrial subunit expression. There appears to be an emerging consensus whereby hemagglutination and proteolytic activities, as well as other functions, cannot be dissociated in *P. gingivalis*. Mutations or selective pressures which affect regulatory or processing genes could explain the simultaneous deficiency in these activities. In this respect, a genetic analysis of our mutants would be of great help in the further identification and characterization of the specific gene or related genes involved in the hemagglutination of *P. gingivalis* as it relates to fimbriation and proteolytic activity and in determining the magnitude of the pleiotropic effects induced by the selective pressure that we used.

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